Title of thesis

Isolation, Screening and Identification of highly efficient α-amylase producing bacteria from vegetable waste of Raipur vegetable market and determine the optimum condition for higher amylase production.

Introduction

Enzymes are capable to act as biocatalyst for a wide variety of chemical reactions. Amylase is one of the most important enzymes in various point of view especially in industries, that catalyses the breakdown of starch into sugar. Amylase is produced by a variety of living organisms, ranging from bacteria to plants & humans. Bacteria & fungi secrete amylases to the outside and inside of their cell to carry out extracellular and intracellular enzyme. When they have broken down the insoluble starch, the soluble end products are produce such as glucose or maltose (Anupama and Jayaraman 2011). This Amylase producing microbes are generally present in degraded atmosphere. Micro-organisms perform their metabolic processes rapidly and with remarkable specificity under ambient conditions. Catalyzed by their diverse enzyme alternatives to harsh chemical technologies has led to extensive exploration of natural microbial diversity to discover enzyme which could function effectively and generate pollution free "dream technologies" in the immediate future. (Sivakumar *et al*., 2012; Anupama and Jayaraman 2011). α-amylases (endo-1,4-α-D-glucan glucanohydrolase [E.C.3.2.1.1]) act on α-1,4 glycosidic bonds of starch to form maltoses, maltotrioses, maltotetrose, glucoses and a mixture of malto-oligosaccharides (Anupama and Jayaraman 2011; Akcan 2011; Bakri *et al*., 2012).

Among microbial, plant and animal enzymes, microbial amylases have immense applications in various fields in world market because of their wide application in starch based industries especially food, textile, paper, detergent, pharmaceutical and baking industries (Anupama and Jayaraman 2011; Amutha and Priya 2011; Akcan 2011; Kaur *et al*., 2012). Example; In food industry amylase use in bread making, to break down complex sugars, such as starch (found in flour), into simple sugars. Modern bread making techniques have included amylases (often in the form of malted barley) into bread improver, thereby making the process faster and more practical for commercial use (Maton *et al.*1993). In medical, Blood serum amylase may be measured for purposes of medical diagnosis. A normal concentration is in the range 21-101 U/L. A higher than normal concentration may reflect one of several medical conditions, including acute inflammation of the pancreas (concurrently with the more specific lipase), but also perforated peptic ulcer,

torsion of an ovarian cyst, strangulation ileuses, macroamylasemia and mumps. Amylase may be measured in other body fluids, including urine and peritoneal fluid (Anupama and Jayaraman 2011; Amutha and Priya 2011; Akcan 2011; Kaur *et al*., 2012).

These α-amylase enzymes account for about 30 % of the world's enzyme production (Akcan 2011; Malle *et al*., 2012; Deb *et al*., 2013). The world market for enzymes remains in excess of $4500 million (Sivakumar *et al*., 2012) presently about US$ 2.7 Billion and increase by 4% annually (Deb *et al*., 2013). Bacteria which can produce the amylase are widely present in nature can easily be screened and tested for the production of amylase (Pokhrel *et al*., 2013). Commercially used alpha-amylase obtained mostly from different types of Bacillus sp. (Pokhrel *et al.*, 2013; Deb *et al.*, 2013; Kumar *et al*., 2013). But amylase enzymes are unstable and loss their property in various conditions like high temperature, pH, and different chemicals used in the reaction and it is still undergoing to find out the bacterial strain which produce more stable amylase enzyme with higher production rate within low cost. So due to this problem we try to screen out new strain from Chhattisgarh region which produce highly stable and large quantity of amylase enzyme.

A brief review of the work already done in the field

Many other researchers are previously worked on this field, some of the following relevant literatures are studied regarding to the isolation, screening and use of microorganisms for α-Amylase production for this research work.

Fogarty (1983) discussed the applications and features of amylases of microbial origin. Gogoi *et al*., (1987) Production, purification and characterization of an α-amylase produced by *Saccharomycopsis fibdigera*. Lonsane and Ramesh (1990) said microorganisms are the most reliable source of enzyme in large scale productions. The major advantages of using microorganisms for production of enzymes are economical production and bulk production capacity. Microbes are also easy to manipulate to obtain enzymes desired characteristics. Achi and Njoku (1992) studied the saccarification of raw starch with amylases isolated from *Bacillus circulans* GRS 313 in calcium alginate beads and studied the process parameters, kinetics and reusability of immobilized enzyme.

Figueira and Hirooka (2000) worked on different types of amylase production medium composition by toxigenic Fungi. Aguilar *et al*., (2000) studied on Purification and characterization of an extracellular α-amylase by *Lactobacillus manihotivorans* LMG

18010^{T}. They found optimum temperature and pH were 55°C and 5.5, respectively. They observed stability of α-amylase was good at pH range from 5 to 6 and when the enzyme was incubated in presence of soluble starch. They found enzyme was sensitive against temperature if the enzyme was incubated at 55°C for 1 hour.

Buzzini and Martini (2002) studied on extracellular enzymatic activity profiles in yeast and yeast like strains isolated from tropical environment. Haq *et al*., (2003) suggested the formulation of medium for production of alpha-amylase by *Bacillus licheniformis.* Akpan and Adelaja (2004) worked on production and stabilization of amylase preparations from rice bran solid medium. Reshmi *et al*., (2006) worked on the enhancement of activity and stability of α-amylase immobilized on alumina. Sivaramakrishnan *et al.,* (2006) were studied various microbial sources for the extracellular production of amylase enzyme.

Liu and Xu (2008) isolate a novel raw starch digesting α-amylase bacteria *Bacillus* sp. YX-1: they worked on the purification and characterization of amylase enzyme. Maximum α-amylase activity (53 U mL^{-1}) was obtained at 45 °C after 44 h of incubation. The enzyme was purified using ammonium sulfate precipitation, ion exchange and gel filtration chromatography, and showed a molecular weight of 56 kDa by SDS-PAGE. This enzyme activity was maximum at pH 5.0, and optimum active temperature was at 40-50°C. de Souza and Magalhães (2010) studied about the application of microbial α-amylase in different types of industries.

Alkando and Ibrahim (2011) isolate a new potential microorganism *Bacillus licheniformis* for α-amylase production and compared the enzymatic activity in two different method and found maximum α-amylase activity of 0.7947 U/mg/ml at pH of 8 in iodine method and (0.024 U/mg/ml) in 3.5.dinitrosalicylic acid (DNS) method. Amutha and Priya (2011) analyzed the effect of pH, Temperature and Metal Ions on Amylase Activity from *Bacillus Subtilis* KCX 006. They found maximum enzymatic activity in stationary phase with optimum temperature of 37°C and in $NiCl_2$, residual activity was found maximum upto117.5%.

Akkaya *et al*., (2012) worked on the immobilization of extracellular thermoalkalophilic amylase produced from bacilli sp. Immobilization of enzyme was performed through its amino groups onto the epoxy rings of magnetic poly glycidyl methacrylate [m-poly (GMA)] beads. They observed that immobilized enzyme activity was showed the stability in higher temperature at 105°C and pH 11.0. Devi *et al.*, (2012) worked

on Immobilization of purified alpha-amylase enzyme which was produced by *Bacillus sp*. They immobilized the enzyme in sodium alginate and studied the stability and activity of immobilized enzyme beads in different pH and temperature. At 60°C and pH 6 the enzyme was incubated for 10 minutes showed maximum enzymatic activity. Jamrath *et al*., (2012) studied about the production of amylases and proteases by *Bacillus caldolyticus* from food industry wastes. In this work, thermostable a-amylase and neutral proteases were produced using the thermophilic strain *Bacillus caldolyticus* DSM 405. Mojsov (2012) studied about different amylases and there industrial application. He studied the way by which amylase enzyme is used in different industries like detergent, beverage, leather, pharmaceutical and paper industries.

Abdel-Fattah *et al*., (2013) worked on Production, Purification, and Characterization of Thermostable *α*-Amylase Produced by *Bacillus licheniformis* Isolate AI20. They studied on constituents of media for higher amylase production and purify with different chromatographic technique. They found optimum pH and temperature is 6-7 and 60 - 80°C respectivily. Asoodeh *et al*., (2013) studied on purification and biochemical characterization of an acidophilic amylase from a newly isolated Bacillus sp. DR90. They also analyzed the effect of different ions and it was observed that enzyme activity was increased by Ba^{2+}, Fe^{2+} and Mg^{2+}, and decreased by Hg^{2+} and Zn^{2+}, while it was not affected by Na^{+}, K^{+}, phenylmethylsulfonyl fluoride and β-mercaptoethanol. Ca^{2+} and EDTA did not have significant effect on the enzyme activity and thermal stability. Kumar *et al*., (2013) worked on thermostable α-amylase enzyme production, purification and characterization from *Bacillus laterosporus*. They analyzed the effect of temperature and pH on purified enzyme and found maximum activity in at optimal conditions of temperature (60°C) and pH 7. They found no effect in the activity of enzyme in presence of Ca^{2+} ions and EDTA and in presence of Mg^{2+} ions, SDS and β-mercaptoethanol activity was redused.

Sani *et al*., (2014) Isolate *Bacillus subtilis* for the production, purification and characterization of α-amylase. There result showed that the partially purified enzyme has specific activity of 0.144±0.019 U/mg, these was increase of 33.5 times than the raw enzyme extract. The optimum pH of the purified enzyme was 6.0, but the enzyme can work in the pH range of 5.0 – 9.0. The optimum temperature of the enzyme was 60°C. Sundarram and Murthy (2014) has studied on the different technique, methods and process parameters for amylase production. They studied the different enzymatic activity determination methods and there applications.

Objectives

The present study is aimed to investigate the following objectives:

1. **Collection of substrate for bacterial strain isolation.**
    - Vegetable waste substrate will be collected from the vegetable market of Raipur Chhattisgarh, for bacterial strain isolation.
2. **Isolation and Screening of bacterial strain.**
    - Isolation of bacterial strain from the vegetable waste sample of vegetable market of Raipur, Chhattisgarh.
    - Screening of bacterial strain producing α-amylase in large quantity through starch hydrolysis test by the visual appearance of large clear zone.
    - Selection of highly α-amylase producing bacterial isolate by quantitative estimation for further analysis.
3. **Identification of most efficient highly α-amylase producing bacteria.**
    - Characterization of highest amylase producing bacterial isolate on the basis of Morphological, Biochemical and finally will be identified up to species level by Molecular characteristics.
4. **Optimization of various parameters for highest α-amylase production from identified bacterial isolate.**
    - The major parameters will be temperature, pH, incubation period and nutrients.
5. **Analysis of data.**
    - Obtained data will be statistically analyzed by using desirable tool.

Noteworthy contribution in the field of proposed work

Gangadharan *et al.*, (2008) worked on the surface methodology for the optimization of α-amylase production by *Bacillus amyloliquefaciens*. Fooladi and Sajjadian (2010) studied on screening the thermophilic and hyperthermophilic bacterial isolate from three Iranian hot-springs for detection of thermostable α- amylase producing strain. They analyzed the produced enzyme activity and found at maximum level of activity at 70°C in the presence of soluble starch (1%) at pH 6. With addition of calcium (10 mM) and peptone (1%) to the mineral medium, shortened the lag period and improved the growth and α-amylase synthesis.

Verma *et al.*, (2011) they worked on production and purification of amylase from bacteria isolated from a waste potato dumpsite in district Farrukhabad of U.P. India. Bozic *et*

al., (2011) worked on production and properties of the highly efficient raw starch digesting α-amylase from a *Bacillus licheniformis* ATCC 9945a.

Sivakumar *et al*., (2012) studied on Amylase Production Using *Bacillus cereus* Isolated from a Vermicompost Site. They studied on the effect of pH, temperature and incubation period to find out proper condition for production of amylase enzyme with the particular bacterial strain. Bakri *et al*., (2012) worked on Isolation and identification of a new *Bacillus* strain for amylase production. The optimum temperature for amylase production was found to be 37°C. Amylase production occurred at pH 3.0-9.0 with a maximum at pH 5.0. The best enzyme activity was observed at pH 6.0 and temperature 70°C. Kaur *et al*., (2012) worked on Isolation, characterization and identification of bacterial strain producing amylase.

Pokhrel *et al*., (2013) studied on isolation, screening and characterization of Promising α-amylase producing bacteria from sewage Enriched soil. In the present study, bacteria were isolated from sewage soil and screened for the production of α-amylase. The bacterial isolate was identified as *Bacillus* sp. They found maximum yield of amylase after 48h of incubation. The optimum pH and temperature for enzyme activity was found to be at pH 7 and 35ºC. Deb *et al*., (2013) studied on Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. The effect of various fermentation conditions on amylase production through shake-flask culture was investigated. Enzyme production was induced by a variety of starchy substrate but corn flour was found to be a suitable natural source for maximum production. Tryptone and ammonium nitrate (0.2%) as nitrogen sources gave higher yield compared to other nitrogen sources. Maximum enzyme production was obtained after 48 hrs of incubation in a fermentation medium with initial pH 9.0 at 42°C under continuous agitation at 150 rpm. The size of inoculum was also optimized which was found to be 1% (v/v). Ravindar and Elangovan (2013) worked on molecular identification of amylase producing bacteria *Bacillus subtilis* and tried to detect optimal condition for amylase production and its activity. They found maximum amylase production (538 U/ml) at 32 °C at pH 7 by the strain SSII2. After partial purification the concentration of alpha amylase was found to be 54.54 mg/L. The maximum amylase enzyme activity was obtained at the beginning of the stationary growth phase.

Andualem (2014) he worked on isolation and screening of amylase producing bacteria and he found thermophilic spore forming *Bacilli* sp. from starch rich soil and check the characteristics of enzyme and its activity using submerged fermentation. He found amylase enzymes optimum activity at 60°C. Mohanasrinivasan *et al*., (2014) worked on process optimization for enhancement of α-amylase Production by submerged and Solid State

Fermentation using *Bacillus pumilus* VITMDS2. In their study, in solid state fermentation maximum amount of α-amylase production (0.956 U/mg) was obtained with inoculum size 9 %, pH 6.5 at 37℃ for 24 h. In Submerged fermentation highest amylase production (0.75 U/mg) was obtained with inoculums size 2 %, pH 7.0 at 30℃ for 72 h. Raul *et al.*, (2014) tried to produce and purify the α-amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation technique.

Proposed methodology during the tenure of the research work

1. **Collection of substrate for bacterial strain isolation.**
    - Vegetable waste substrate will be collected in every month through out one year from the vegetable market of Raipur Chhattisgarh, for bacterial strain isolation.
    - Waste vegetable substrate will be collected in a clean flask containing sterile distilled water from the Raipur vegetable market and stored in 4°C for bacterial strain isolation.
2. **Isolation and Screening of bacterial strain.**
    - Collected vegetable waste sample in water mixed by gently shaking will be used for the isolation of bacterial strain through serial dilution method in pre poured NAM (Nutrient Agar Medium) plates (Prescott and Harley 2002).
    - Isolated bacterial strain then will be pure cultured in NAM plates, after that screening of amylase enzyme producing bacterial strain will be performed in SAM (Starch Agar Medium) plate through starch hydrolysis test by appearance of clear zone with flooding of iodine solution (Prescott and Harley 2002).
3. **Selection of highly α-amylase producing bacterial isolate.**
    - Selection of highly α-amylase producing bacterial isolate by quantitative estimation will be performed through amylase enzymatic activity. First α-Amylase will be produced from amylase producing bacterial isolate in modified production medium as per Gogoi *et al*., (1987). Then produced enzyme will be separated from bacterial culture through centrifugation at 10,000 rpm for 15-20 minutes. This crude enzyme extract further will be used for quantitative estimation by the modified 3, 5-dinitrosalicylic acid (DNS) method. (Miller 1959; Aguilar *et al*., 2000; Anto *et al*., 2006; Bakri *et al*., 2012; Deb *et al*., 2013).

4. **Identification of most efficient highly α-amylase producing bacteria.**
    - Characterization of highest amylase producing bacterial isolate will be on the basis of Morphological, Biochemical (Prescott and Harley 2002) and finally will be identified up to species level by Molecular characteristics.
5. **Optimization of various parameters for highest α-amylase production from identified bacterial isolate.**
    - Culture condition will be standardize for high amylase enzyme production by the optimization of different parameters pH, temperature, incubation period and nutrients for the maximum production of amylase enzyme (Gogoi *et al*., 1987; Reshmi *et al*., 2006; Balkan and Ertan 2007; Sindhu *et al*., 2009; Asoodeh *et al*., 2013; Sani *et al*., 2014).

Expected outcome of the proposed work

α-Amylase is the most common enzyme used worldwide in many industries for various purposes. Present production quantity in the world is not sufficient to fulfill the need of amylase enzyme. Bacterial strains which are now used for commercial production do not fulfill the amount of enzyme and quality as per need. From this investigation new bacterial strain will be isolate which produce high amount of stable amylase enzyme. Proper optimized condition which increases the production of amylase enzyme from particular substrate and microbes will be found. A novel technique will be developed for the amylase enzyme production which will be cheap and easier.

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Supervisor

(Prof. K. L. Tiwari)

Dr. K. L. Tiwari
Former Prof. & Head
School of Studies in Biotechnology
Pt. Ravishankar Shukla University
RAIPUR-492 010 (C. G.)

Co-supervisor

(Prof. S. K. Jadhav)

Dr. S. K. JADHAV
Professor & Head
SOS in Biotechnology
Pt. Ravishankar Shukla University
RAIPUR-492 010 (C. G.)

Signature of Candidate

(Jai Shankar Paul)

Forwarded

Chairman, DRC

Chairman (DRC)
SOS in Biotechnology
Pt. Ravishankar Shukla University
Raipur-492010 (C. G.)